

Applicants: Peter David East and Susan Elizabeth Brown
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Amendments to the specification

Please replace the paragraph on page 19, lines 16-29, with the following amended paragraph:

A polynucleotide of the present invention may selectively hybridise to a polynucleotide that encodes a peptide of the present invention under high stringency. Furthermore, oligonucleotides of the present invention have a sequence that hybridizes selectively under high stringency to a polynucleotide of the present invention. As used herein, high stringency conditions are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% NaDODSO₄ at 50°C; (2) employ during hybridisation a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin, 0.1% Ficoll FICOLL™ (a neutral, highly branched, high-mass, hydrophilic polysaccharide), 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 g/ml), 0.1% SDS and 10% dextran sulfate at 42°C in 0.2 x SSC and 0.1% SDS.

Please replace the heading on page 25, line 4, with the following amended heading:

Transgenic ~~Non~~ Non-Human Animals

Please replace the paragraph beginning on page 31, line 35 to page 32, line 13, with the following amended paragraph:

Two crude hemolymph samples from different *G. mellonella* immunizations were processed by C18 solid phase extraction.

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The thawed hemolymph (1.4 or 4.8 ml) was diluted into an equal volume of 0.1% trifluoroacetic acid (TFA), and shaken on ice for 30-45 min. The samples were centrifuged at high speed for 10 min and the supernatant removed. The first sample (from 1.4 ml of hemolymph) was precipitated with 20% acetonitrile/0.05% TFA, and re-centrifuged for 5 min at high speed. The supernatant was loaded onto three C18 solid phase extraction cartridges (Maxi-Clean MAXICLEAN™, 300 mg cartridges, Alltech) equilibrated in 20% acetonitrile/0.05% TFA. Each cartridge was washed with 20% acetonitrile/0.05% TFA, and eluted with 1 ml of 60% acetonitrile/0.05% TFA. The second sample (from 4.8 ml of hemolymph) was loaded onto three C18 solid phase extraction cartridges (Maxi-Clean, 900 mg cartridges, Alltech) equilibrated in 0.05% TFA. The cartridges were washed with 0.05% TFA and eluted stepwise with 3 ml 20% acetonitrile/0.05% TFA followed by 3 ml 60% acetonitrile/0.05% TFA. Samples (1 ml) from the 60% acetonitrile/0.05% TFA elution were dried in a Speedvac (Savant) and resuspended in 100 µl water. The samples were tested against *E. coli*, *M. luteus* and various fungi using the plate assay described above.

Please replace the paragraph on page 32, lines 16-21, with the following amended paragraph:

The crude hemolymph sample was purified by reverse phase HPLC on a Beckman Gold system monitoring absorbance at 225 or 215 nm. Sample (1.4-1.8 ml) was loaded onto a Jupiter C18, 5 µm, 300 Å, 250 x 10mm semi-prep column (Phenomenex) equilibrated in solvent A (2% acetonitrile, 0.065% TFA), and eluted with a gradient from 0-70% solvent B (95% acetonitrile, 0.05% TFA) over 70 min at 5 ml/min. 500 µl of each 5 ml fraction was dried in the a vacuum concentrator (Speedvac SPEEDVAC™), resuspended in 10 µl water, and tested for activity against *E. coli*, *M. luteus* and *F. graminearum*, as described above.

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Please replace the paragraph on page 32, lines 22-33, with the following amended paragraph:

For Gm-moricinA, the fraction of interest was diluted in an equal volume of 0.05% TFA and loaded onto a Prosphere PROSPHERE™ C18, 5 µm, 300 Å, 250 x 4.6mm analytical column (Alltech) equilibrated in 10% solvent B on the HPLC. The column was eluted with a gradient of 10-50% B running over 60 min at 1ml/min. 200 µl of each 1.8 ml fraction was dried in the Speedvac vacuum concentrator (SPEEDVAC™), resuspended in 10 µl water and tested for activity against *E. coli*, *M. luteus* and *F. graminearum*. The fraction of interest was diluted in an equal volume of 0.05% TFA and loaded onto a Macrosphere MACROSPHERE™ C8, 5 µm, 300 Å, 250 x 4.6mm analytical column (Alltech) equilibrated in 15% solvent B on the HPLC. The column was eluted with a gradient of 15-55% solvent B running over 60 min at 1ml/min. 300 µl of each 1.8 ml fraction was dried in the Speedvac, resuspended in 10 µl water and tested for activity against *E. coli*, *M. luteus* and *F. graminearum*.

Please replace the paragraph beginning on page 32, line 34 to page 33, line 14, with the following amended paragraph:

For Gm-moricinB, the fraction of interest was diluted in an equal volume of 0.05% TFA and loaded onto a Prosphere PROSPHERE™ C18, 5 µm, 300 Å, 250 x 4.6mm analytical column (Alltech) equilibrated in 15% solvent B on the HPLC. The column was eluted with a gradient of 15-65% B running over 75 min at 1ml/min. 200 µl of each 1.8 ml fraction was dried in the Speedvac vacuum concentrator (SPEEDVAC™) resuspended in 10 µl water and tested for activity against *E. coli*, *M. luteus* and *F. graminearum*. The fraction of interest was diluted in an equal volume of 0.05% TFA and loaded onto a Macrosphere MACROSPHERE™ C8, 5 µm, 300 Å, 250 x 4.6mm analytical column (Alltech) equilibrated in 15% solvent B

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on the HPLC. The column was eluted with a gradient of 15-55% solvent B running over 60 min at 1ml/min. 200 μ l of each 1.8 ml fraction was dried in the Speedvae vacuum concentrator (SPEEDVAC™), resuspended in 10 μ l water and tested for activity against *E. coli*, *M. luteus* and *F. graminearum*. The fraction of interest was diluted in an equal volume of 0.05% TFA and loaded onto a μ RPC C2/C18, 3 μ m, 100 \times 2.1mm analytical column (Amersham Biosciences) equilibrated in solvent A running on a SMART system (Amersham Biosciences) monitoring at 215, 254 and 280 nm. The column was eluted with a gradient of 0-100% solvent B running over 25 min at 200 μ l/min. 50 μ l of each 200 μ l fraction was dried in the Speedvae vacuum concentrator (SPEEDVAC™), resuspended in 3 μ l water and tested for activity against *F. graminearum*.

Please replace the paragraph on page 33, lines 15-25, with the following amended paragraph:

For Gm-moricinC1, the fraction of interest was diluted in an equal volume of 0.05% TFA and loaded onto a ProSphere PROSPHERE™ C18, 5 μ m, 300 Å, 250 \times 4.6mm analytical column (Alltech) equilibrated in 10% solvent B on the HPLC. The column was eluted with a gradient of 10-50% B running over 60 min at 1ml/min. 400 μ l of each 1.8 ml fraction was dried in the Speedvae vacuum concentrator (SPEEDVAC™), resuspended in 10 μ l water and tested for activity against *F. graminearum*. The fractions of interest were pooled and diluted in an equal volume of 0.05% TFA and loaded onto the C2/C18 column. The column was equilibrated in solvent A running on the SMART system and was eluted with a gradient of 0-100% solvent B running over 25 min at 200 μ l/min while monitoring at 215, 254 and 280 nm. Fractions were collected by peak detection and tested directly against *F. graminearum*.

Please replace the paragraph on page 33, lines 28-36, with the

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following amended paragraph:

The fractions of interest were analysed on a ~~Voyager Elite~~ VOYAGER-ELITE™ MALDI-TOF mass spectrometer (Perseptive Biosystems) using 0.5 µl of sample plus 0.5µl of matrix. For linear mode spectra the matrix was sinapinic acid and the standard was a mixture of cecropin A and myoglobin, and for reflector mode spectra the matrix was α-cyano-4-hydroxycinnamic acid and the standard was a tryptic digest of bovine serum albumin. For N-terminal amino acid sequencing the purified peptides were dried onto fibre glass disks and subject to Edman degradation using a ~~Precise~~ PROCISE™ Model 492 Protein Sequencer (Applied Biosystems), in accordance with the manufacturers instructions.

Please replace the paragraph on page 35, lines 13-16, with the following amended paragraph:

Total RNA was isolated using Trizol TRIZOL™ reagent (Astral Scientific) (Guanidinium thiocyanate phenol-chloroform extraction). Briefly, approximately 500 mg of frozen fat body tissue was resuspended in 1mL of Trizol TRIZOL™ reagent and homogenised in a Polytron POLYTRON™ tissue homogeniser.

Please replace the paragraph on page 35, lines 28-32, with the following amended paragraph: .

A cDNA library was prepared from approximately 5 µg of mRNA using a Lambda UniZap UNIZAP™ cDNA synthesis and cloning system (Stratagene). Purified cDNA (approx. 20 ng) was ligated to 1µg of vector DNA and packaged with Gigapack GIGAPACK® III Gold packaging extract (Stratagene) to yield a cDNA library with a titre of 5 x 10⁵ plaque forming units per mL.

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Please replace the paragraph on page 36, lines 13-23, with the following amended paragraph:

PCR products of the expected size were excised from acrylamide gels and the DNA was eluted in ammonium acetate buffer and recovered by ethanol precipitation. The purified DNA was ligated into the cloning vector pGEM-T™ EASY™ T_{easy} (Promega) and transformed by electroporation into *E. coli* DH10B cells. Resultant bacterial colonies were screened for inserts by PCR using primers based on the T7 and SP6 promoter sequences flanking the multiple cloning site of the vector. For each of the Gm-morcinA and Gm-morcinB PCR products, several clones containing inserts of the expected size were sequenced on both strands and the deduced protein sequence was used to verify the clones as true Gm-morcinA and Gm-morcinB cDNA products. A representative clone was selected for each morcin cDNA type for subsequent use in library screening.

Please replace the paragraph on page 39, lines 15-21, with the following amended paragraph:

The amino acid sequence of Gm-morcinC1 obtained by peptide sequencing was used to design degenerate primers (Gm3-1 and Gm3-2, see Table 5) in order to isolate the gene by PCR from the *G. mellonella* cDNA library. Sequencing of PCR products identified two forms of the Gm-morcinC gene. Specific primers (GmC1-F, GmC1-R, GmC2-F and GmC2-R, see Table 5) were designed which when used in nested PCR with vector primers based on pBluescript~~LUESCRIPT~~™ SK phagemid (Stratagene) would distinguish between the two forms of the gene.

Please replace the paragraph on page 45, lines 31-35, with the following amended paragraph:

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From maintenance stocks, cores were taken and used to inoculate 500 ml Potato Dextrose Broth (PDB). Flasks were incubated on a shaker for 7 days at 28 °C. The inoculum was drained through Miraeloth MIRACLOTH™ filtration material prior to quantification with a haemocytometer. The spores were diluted with sterile distilled water and used to inoculate *Arabidopsis* strains.

Please replace the paragraph on page 47, lines 8-19, with the following amended paragraph:

Protein electrophoresis was performed using 10% Bis-Tris NuPAGE™ Novex pre-cast polyacrylamide gels (Invitrogen) and a MES running buffer, as recommended by the manufacturer. Western blot transfers were performed using a 0.2 µm Trans-Blot^{TRANSBLOT™} nitrocellulose membrane (BioRad) on a Neavablot^{OVABLOT™} semi-dry blotter at 0.8 mA/cm² in transfer buffer (25 mM Bicine, 25mM Bis-Tris, 1 mM EDTA, pH 7.2) containing 20% methanol. The membrane was processed at room temperature in TBS containing 0.1% Tween-20 using three 5 min washes between all steps. The steps used were an overnight block with 3% BSA, a 1 hour incubation with the peptide antiserum (1/250-1/500 dilution) and a 1 hour incubation with anti rabbit IgG alkaline phosphatase conjugate (Sigma, 1/30000 dilution). The blots were visualized using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega) in substrate buffer (100 mM Tris-Cl, 5 mM MgCl₂, 100 mM NaCl, pH 9.5).

Please replace the paragraph beginning on page 47, line 27 to page 48, line 2, with the following amended paragraph:

The Gm-moricinA peptide was expressed in Sf21 cells using a recombinant baculovirus constructed using GATEWAY™ technology (Invitrogen). Primers were designed containing

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the *attB1* and *attB2* recognition sequences linked to eukaryotic control regions and Gm-moricinA-specific sequences (Lm1attB1, 5'-attB1-TCGAAGGAGATGCCACCATGAAGTTACAGGAATATTCTTCA-3' (SEQ ID NO:45) and Lm2attB2, 5'-attB2-TTAGTGCCTCTGTTTAATGTGTTCATAGAC-3' (SEQ ID NO: 46)). These primers were used with *Pfx* polymerase (Invitrogen) to amplify a Gm-moricinA-*attB* PCR product. The PCR product (100 fmol) was shuttled via the pDONR201 entry vector (100 fmol) into the pDEST-8 baculovirus destination vector according to the manufacturer's instructions. Transformants were selected on ampicillin plates and plasmids prepared using the FastPlasmidASTPLASMID™ mini plasmid purification kit (Eppendorf). Positive transformants were confirmed by PCR, restriction enzyme digestion and sequence analysis.